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ABROGATION OF ETOPOSIDE-MEDIATED CYTOTOXICITY BY CYCLOHEXIMIDE

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Abstract—The antitumor agent etoposide interacts with DNA topoisomerase II to produce a unique form of DNA–enzyme intermediate referred to as a "cleavable complex". These drug-induced DNA strand breaks initiate poorly defined cell processes which result in lethality. To explore the mechanism of etoposide cytotoxicity, we studied the effect of protein synthesis inhibitor on Balb/C 3T3 fibroblasts and CCRF-CEM and L1210 leukemia cells by exposing these cell lines to cycloheximide for various periods of time prior to etoposide challenge. Cycloheximide alone during these periods of exposure was not cytotoxic; however, it conferred increasing cytoprotection from etoposide in a time-dependent fashion when it preceded etoposide. Although cycloheximide did cause a decrease in enzyme content and in etoposide-induced DNA cleavage of Balb/C 3T3 and the CCRF-CEM cell lines, cytoprotection by cycloheximide could not be accounted for completely by these phenomena since, in L1210 cells, cytoprotection was observed without significant change in DNA cleavage or enzyme content. Cycloheximide diminished DNA synthesis as well as protein synthesis. However, DNA synthesis resumed within 6 hr after removal of cycloheximide, in spite of the fact that cytoprotection persisted. Cycloheximide did not alter cell cycle distribution as measured by flow cytometry. Our data, therefore, clearly demonstrate that cycloheximide can diminish the cytotoxic potential of etoposide-mediated topoisomerase–DNA complexes. Elucidiation of the mechanism by which cytoprotection occurs should shed light on the basis for the cytotoxic effect of topoisomerase II-active drugs.

The nuclear enzyme DNA topoisomerase II serves as an important intracellular target for certain classes of anticancer agents such as intercalating agents [1, 2] and epipodophyllotoxins [3–5]. Much remains to be learned, however, regarding the mechanism by which drug–enzyme interaction results in cell death. Current evidence suggests that, in the presence of drug, topoisomerase II forms a stable complex with DNA, referred to as a "cleavable complex", which, upon protein denaturation reveals single- or double-strand breaks in the DNA in which the enzyme is covalently linked to the 5′ termini of the break site [6, 7]. These cleavable complexes are rapidly dissociated upon drug removal [8–10], yet their brief presence within the cell appears sufficient to result in cell death. This suggests that the formation of cleavable complex may initiate a cascade of toxic events which have yet to be characterized.

In previous investigations of the mechanism of etoposide (VP-16) cytotoxicity and its relationship to topoisomerase II, we reported that quiescent Balb/C 3T3 (A31) fibroblasts have a low intracellular content of topoisomerase II compared to logarithmically growing cells and are relatively drug resistant [11]. Upon serum stimulation, enzyme content rises beginning in early S phase and peaks in G_2/M. Interestingly, we found that sensitivity to the cytotoxic effects of etoposide is maximal during late S phase, in spite of the fact that drug-induced DNA cleavage is maximal several hours later. Furthermore, the protein synthesis inhibitor cycloheximide could abruptly (within 3 hr) abort not only the increase in enzyme content but also completely block the cytotoxic action of the drug. Other investigators have shown that protein synthesis inhibition can protect cells from cytotoxic agents which putatively act via topoisomerase II [12–14]. In this report, we studied more extensively the nature of the interaction between cycloheximide and topoisomerase-active cytotoxic agents. We examined a variety of asynchronously growing malignant cell lines with respect to etoposide cytotoxicity and DNA cleavage following pretreatment with cycloheximide. Our results indicate that, while cycloheximide can reduce enzyme content in some cell lines, in others, cytoprotection is observed without alteration in etoposide-induced cleavable complex formation and without discernible change in enzyme content.

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† Abbreviations: DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; and PBS, phosphate-buffered saline (pH 7.4).

MATERIALS AND METHODS

Materials. Cell culture medium and fetal calf serum (FCS) were from Gibco Laboratories (Grand Island, NY). Etoposide was supplied by Bristol Laboratories (Wallingford, CT) and dissolved into DMSO† as a 40 mM stock solution. [^{3}H]Etoposide

was purchased from Moravek Biochemicals (Brea, CA). Radioisotopes and Aquasure were from New England Nuclear (Boston, MA) or Amersham (Arlington Heights, IL); cycloheximide was from the Sigma Chemical Co. (St. Louis, MO) and tetrapropylammonium hydroxide from the RSA Corp. (Ardsdale, NY). All other materials were reagent grade from Bio-Rad Laboratories (Rockville Centre, NY), the Fisher Scientific Co. (Orlando, FL) or Pharmacia, Inc. (Piscataway, NJ).

Cell culture. Balb/C 3T3 (A31) fibroblasts were grown as monolayer in Dulbecco's Minimum Essential Medium (MEM) plus 10% FCS. Human lymphoblast CCRF-CEM cells and mouse leukemia L1210 cells were grown in suspension in α-MEM plus 10% FCS and RPMI 1630 plus 20% FCS respectively. All cell culture took place at 37°, and all media were supplemented with 3 mM glutamine, penicillin (100 I.U./ml), and streptomycin (100 μg/ml).

DNA alkaline elution. Alkaline elution was performed using a low-sensitivity assay as previously described [15]. Cells were washed twice with 1 × PBS at 4° before alkaline elution.

Drug treatments. For the A31 cell line, cells were seeded at 2×10^5 cells/T_{25} flask 42 hr prior to the experiment. L1210 and CCRF lines were maintained in log-growth phase and treated at a cell density of 10^5/ml. All cells were incubated with cycloheximide for various periods of time prior to etoposide challenge. Etoposide was added in the final hour of each experiment, after which cells were washed twice with warm medium. For monolayer cultures, cells were then trypsinized, and viable cells, as determined by Trypan blue dye exclusion, were seeded quantitatively into 100-mm culture dishes with 10 ml of medium. Cytotoxicity was assayed by colony formation [16]. Suspension cells were seeded into tubes with 0.1% agar. Survival was expressed as a percent of DMSO-treated controls.

Flow cytometry. DNA content was determined by methods previously described [17].

Thymidine and leucine incorporation studies. DNA and protein syntheses were assayed by incorporation of [^{14}C]thymidine and tritiated leucine at 37° for 30 min into trichloroacetic acid (TCA) insoluble material.

Gel electrophoresis and immunoblotting. Following cycloheximide treatment, cells were washed with serum-free medium and then lysed in an SDS buffer (50 mM Tris, pH 6.8, 10% glycerol, 1 mM PMSF, 5% β-mercaptoethanol, 0.01% bromophenol blue, and 2% SDS). Electrophoresis was carried out in 7.5% polyacrylamide gel with a 4.5% stacking gel. Transferring to nitrocellulose, probing with a mouse anti-serum to human topoisomerase II, and developing the immunoblot have been described previously [11].

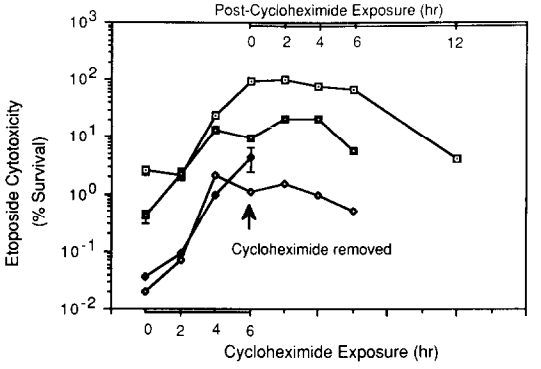

Fig. 1. Etoposide cytotoxicity studies of A31 (⊡, 40 μM), CCRF-CEM (◆, 40 μM) and L1210 (□, 10 μM; ◇, 40 μM) cells following incubation with 50 μM cycloheximide for 2, 4 or 6 hr or at various times after cycloheximide removal. Etoposide (10 or 40 μM) was added at the final hour of each experiment. The values shown are the means of nine data points from three independent experiments. Standard deviations were less than 2% of the means unless shown.

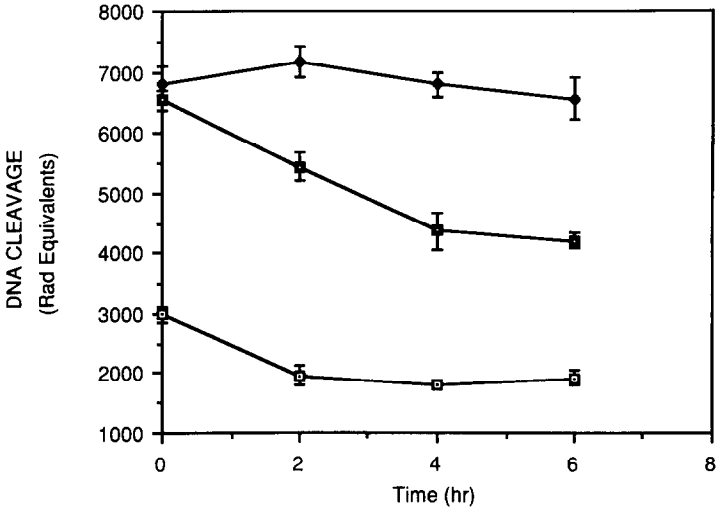

Fig. 2. Effect of cycloheximide on cell sensitivity to etoposide-induced DNA cleavage. A31 (⊡), CCRF (□) and L1210 (◆) cells were exposed to 50 μM cycloheximide for 2, 4 or 6 hr. Etoposide (40 μM) was added for the final hour. Cells were then assayed for DNA cleavage by the alkaline elution technique. Values shown are mean ± SD, N = 3.

RESULTS

The effect of cycloheximide on etoposide cytotoxicity in three different cell lines was determined by incubating the cells in 50 μM cycloheximide for various periods of time prior to adding etoposide (Fig. 1). The etoposide treatment overlapped the last hour of cycloheximide exposure. In each of the three cell lines, a significant reduction in etoposide cytotoxicity was observed which became more profound with increasing duration of cycloheximide exposure. Although etoposide was more toxic to L1210 and CCRF cells than to the fibroblast line, the slope of protection with respect to time was similar in all lines. In the A31 cell line, complete protection was observed after 6 hr of cycloheximide exposure. To determine the duration of the cycloheximide effect, both A31 and L1210 cells were washed free of cycloheximide after a 6-hr exposure. Etoposide was added for 1 hr at various times thereafter. The protective effect of cycloheximide remained for at least 6 hr in both cell lines, although there was clear evidence of a decreasing effect in the A31 line between 6 and 12 hr. It should be noted that cycloheximide alone was not cytotoxic under the conditions of these experiments, and uptake of drug as assayed by net accumulation of [^{3}H]etoposide [18] in the cells was not affected during these periods (data not shown).

The cytotoxicity of etoposide generally correlates with cleavable complex formation as measured by DNA breaks using the alkaline elution technique [9, 19]. To understand the basis for cycloheximide protection, we analyzed DNA breakage induced by etoposide following various periods of cycloheximide exposure (Fig. 2). As was the case for cytotoxicity, A31 fibroblasts were inherently less sensitive to the DNA cleavage effect of etoposide. There was a reduction in strand breakage observed within 2 hr of cycloheximide exposure which did not change with a further duration of exposure. A decrease in etoposide-induced DNA cleavage was also observed in CCRF-CEM cells, although this cell line was initially much more sensitive to etoposide than were the A31 cells. In contrast, no significant change in DNA breakage was observed in L1210 cells for up to 6 hr of cycloheximide exposure. Cycloheximide itself had no effect on DNA cleavage (data not shown).

DNA cleavage by etoposide is mediated by the nuclear enzyme DNA topoisomerase II [5, 19]. We have reported previously that sensitivity to etoposide can be altered dramatically by changes in intracellular content of topoisomerase II [11, 20]. Therefore, enzyme content was measured in whole cell lysates using a mouse antiserum to HeLa topoisomerase II (Fig. 3). There was a substantial decrease of enzyme in the A31 cells between hr 4 and 6 of cycloheximide exposure. A smaller decrease in content in the CCRF cells was observed during the last few hours of cycloheximide exposure. No significant change in enzyme content occurred in L1210 cells.

Inhibition of protein synthesis by cycloheximide also results in inhibition of DNA synthesis [21, 22]. To compare the time course of these two events with that of protection from etoposide cytotoxicity, L1210 cells were incubated with 50 μM cycloheximide for up to 6 hr. In some experiments, cells were then washed free of cycloheximide and resuspended in warm drug-free medium for another 6 hr in order to ascertain the recovery of macromolecular synthesis. Incorporation of radiolabeled thymidine and leucine was promptly and dramatically inhibited by cycloheximide (Fig. 4). After the drug was washed off, however, both recovered rapidly. Thymidine incorporation, in fact, reached pretreatment levels by 6 hr

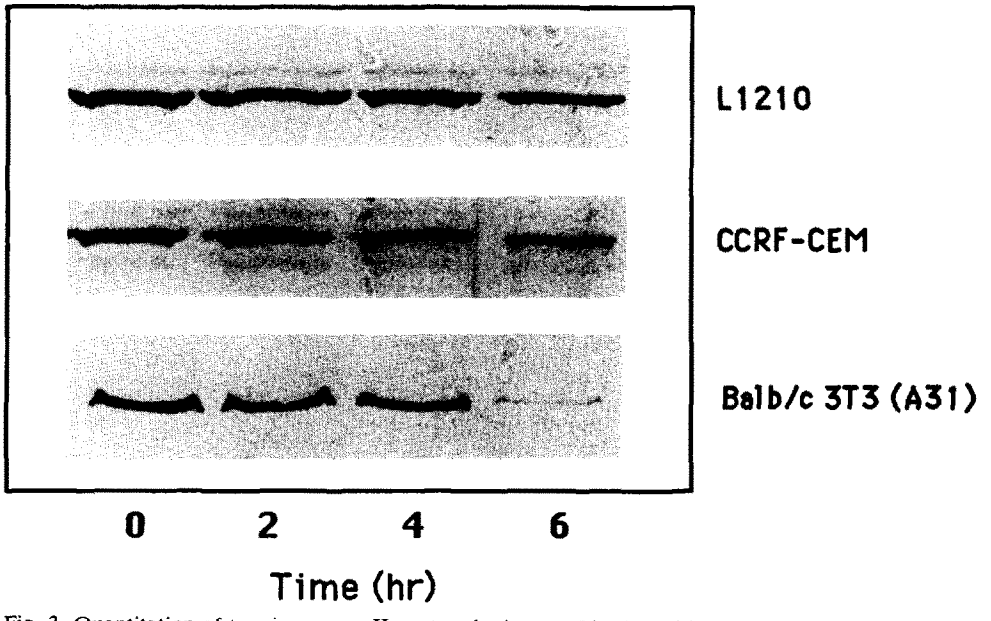

Fig. 3. Quantitation of topoisomerase II content by immunoblotting with mouse antiserum to human topoisomerase II. Cells were incubated with cycloheximide for 0, 2, 4 or 6 hr and then lysed in an SDS buffer, electrophoresed, and immunoblotted. The bands shown correspond to a molecular weight of 168 kD.

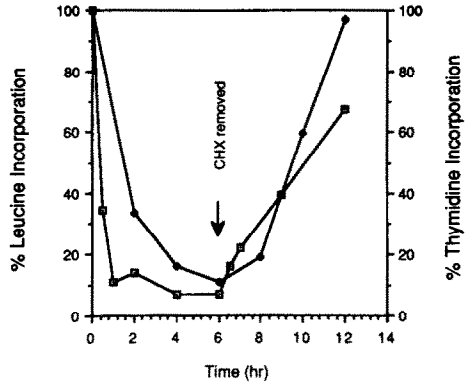

Fig. 4. Effect of cycloheximide on radiolabeled thymidine and leucine incorporation in L1210 cells. Cells were incubated with 50 μM cycloheximide for up to 6 hr and then washed free of drug with fresh warm medium. Incorporation of [^{3}H]leucine (⊡) and [^{14}C]thymidine (◆) into TCA-insoluble material was assayed at various times following the addition or removal of cycloheximide. Data are expressed as percent of control values obtained in cells not exposed to cycloheximide. Control values: [^{3}H]leucine, 5×10^4 cpm; and [^{14}C]thymidine, 6×10^5 cpm.

after washing off cycloheximide, while recovery of leucine incorporation was not quite complete by this time. Thus, DNA synthesis has recovered at a time

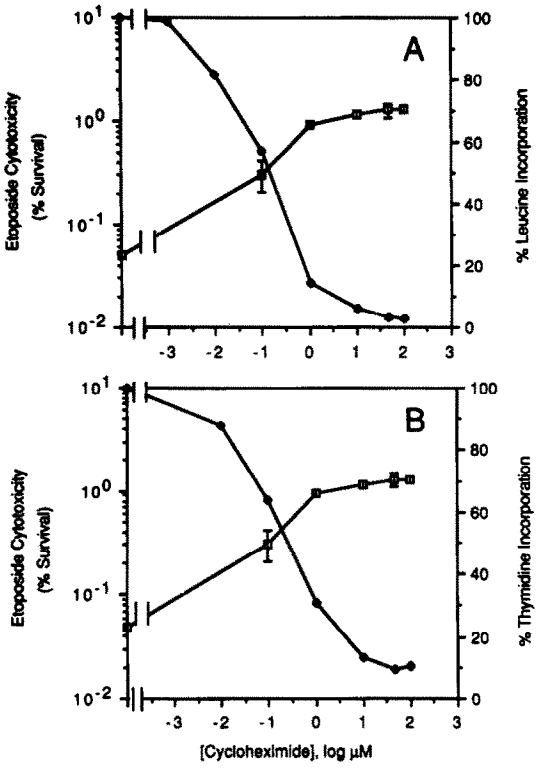

Fig. 5. Comparison of cycloheximide cytoprotection from etoposide with reduction in (A) leucine and (B) thymidine incorporation in L1210 cells at various cycloheximide concentrations. Cycloheximide exposure was for 4 hr, and the etoposide concentration was 40 μM at the final hour. Etoposide cytotoxicity (⊡) is presented as percent survival and precursor incorporation (◆) as the percent reduction compared to untreated cells.

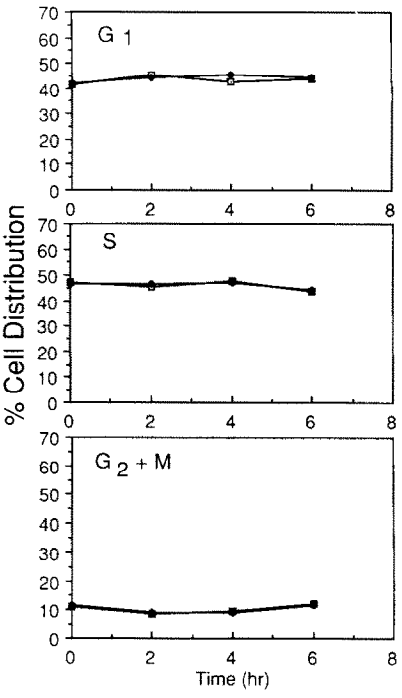

Fig. 6. Effect of cycloheximide on cell cycle progression of log phase L1210 cells as measured by flow cytometry. Cells were incubated with cycloheximide for 2, 4 or 6 hr prior to analysis. Key: cycloheximide-treated cells (◆), and untreated cells (⊡).

when the cells are still protected from etoposide cytotoxicity. To better correlate inhibition of thymidine and leucine incorporation with the cytoprotective effect of cycloheximide, we treated L1210 cells with various concentrations of the protein synthesis inhibitor for 4 hr and then assayed cells for macromolecular synthesis inhibition and etoposide cytotoxicity. These results are shown in Fig. 5 and

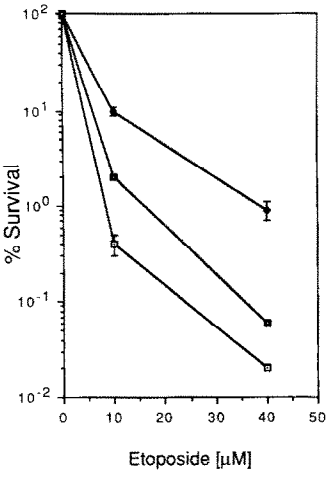

Fig. 7. Comparison of the effects of cycloheximide and aphidicolin on VP-16 cytotoxicity. L1210 cells were incubated with 17 μM aphidicolin (□) or 50 μM cycloheximide (◆) for 6 hr. For the last hour of treatment, etoposide was added to the cells, and following removal of both drugs, clonogenicity was assayed. Control cells (⊡) received etoposide only.

indicate that maximal cytoprotection correlates with a high degree of inhibition of macromolecular synthesis.

This laboratory and others [11, 23–25] have shown that cytotoxicity to etoposide is greater during late S phase than during other periods of the cell cycle. Since cycloheximide obviously perturbed important cellular events (Fig. 4), we ascertained the effects of protein synthesis inhibition on cell cycle distribution using flow cytometry. As shown in Fig. 6, no significant effect was observed on this parameter during 6 hr of cycloheximide exposure.

To determine if the effects of cycloheximide were simply a result of DNA synthesis inhibition, L1210 cells were pretreated with aphidicolin, a DNA polymerase alpha inhibitor, for 6 hr prior to exposure to etoposide. Under these conditions, DNA synthesis was inhibited by greater than 95%. Although some cytoprotection was observed (Fig. 7), the difference was marginal compared to that of cycloheximide.

DISCUSSION

In previous work, we have demonstrated that exposure of cells to cycloheximide during S phase in synchronized A31 cells results in an abrupt loss of intracellular topoisomerase II content and a commensurate loss of etoposide cytotoxicity. In the current experiments, however, we found that the effects of cycloheximide were not based solely on alterations of enzyme content or activity. This was most clearly demonstrated in the case of L1210 cells in which cytoprotection was not attended by any significant alteration in etoposide-induced DNA cleavage (Fig. 2) or in intracellular enzyme content (Fig. 3). These results are somewhat in contrast to those of A31 and CCRF-CEM cells in which decreases in DNA cleavage and enzyme content were observed. An examination of the time course, however, would suggest that even in these two lines cytoprotection was not fully accounted for by these changes. Cycloheximide cytoprotection increased throughout the 6-hr period in A31 and CCRF-CEM cells even though the effects on strand breakage occurred early in the course.

Cytoprotection from topoisomerase II-active chemotherapy agents by protein synthesis inhibitors has been reported previously [13, 14, 26]. Teodori *et al.* [26] have shown that the protein synthesis inhibitor anguidine protects Chinese hamster ovary (CHO) cells from adriamycin cytotoxicity. This effect was observed only if anguidine preceded adriamycin. In their subsequent work [13], the effects of anguidine were not seen in plateau phase CHO cells. The authors interpreted their results as indicating that the protective effect of anguidine is based on inhibition of cell cycle progression. Charcosset *et al.* [14] examined the effect of cycloheximide on 9-OH-ellipticine cytotoxicity in Chinese hamster lung fibroblasts. As previously reported for anguidine, these investigators found that significant cytoprotection was observed when cells were exposed to cycloheximide prior to the ellipticine derivative. Protection was not seen, however, when the cycloheximide was employed following treatment with 9-OH-ellipticine. Interestingly, these authors proposed that the cycloheximide effect may be due to an essential protein such as topoisomerase II which must be present at the time of drug treatment for cytotoxicity to occur.

Based on our results, at least four possible mechanisms could be formulated to interpret the cytoprotective effect of cycloheximide. First, it is possible that, despite the fact that its general effect is that of net protein synthesis inhibition, cycloheximide may be cytoprotective because of the induction of proteins which confer resistance. Sorrentino *et al.* [27] have shown that doses of cycloheximide which decrease protein synthesis by at least 50% in either human or murine cells "induce" the synthesis of proteins which exhibit similarities to "stress-induced proteins". A recent report even suggested that such proteins may be related to cytoprotection from adriamycin following hypoxic insult or glucose deprivation [28]. While there is no direct evidence to support this possibility, it is one which bears investigation. The second possible mechanism for cycloheximide cytoprotection is that the expression of drug lethality requires the presence of certain unidentified proteins which would be depleted by cycloheximide pretreatment. The effects of etoposide and other topoisomerase II-active agents have been compared mechanistically to that of the antibiotic nalidixic acid, an inhibitor of DNA gyrase. Nalidixic acid has been shown to induce the "SOS" repair and heat-shock responses in bacteria [29, 30]. Indeed, there is evidence that the SOS response may enhance nalidixic acid cytotoxicity. Importantly, protein synthesis is required for full expression of the SOS response. While the SOS system has not been identified clearly in mammalian cells, it is likely that a similar mechanism exists. Kupfer *et al.* [37] suggested that a similar response may play a role in etoposide cytotoxicity. Interference with such a response by protein synthesis inhibition could alter the expression of lethality following cleavable complex formation. Pommier *et al.* [32] demonstrated that cell killing of intercalating agents correlates well with drug-induced double-strand DNA breaks, sister chromatid exchange, and mutations. This provides the basis for the third possible explanation for cytoprotection by cycloheximide. Ikeda [33, 34] observed that DNA gyrase and phage T_4 topoisomerase II can mediate DNA recombination events by a novel mechanism involving exchange of enzyme subunits. In the case of gyrase, recombination is stimulated in the presence of nalidixic acid. If, as suggested by Pommier, drug stimulation of sister chromatid exchange occurs by a similar mechanism involving topoisomerase II, it is possible that less homologous forms of recombination may also be occurring undetected. Cycloheximide can profoundly inhibit sister chromatid exchange [35] and, therefore, perhaps illicit recombination events as well. Finally, as was suggested for the effects of anguidine [13, 26], it is possible that the protective action of cycloheximide is based on inhibition of cell cycle traverse. That this is not the case in our system is suggested by the rapid recovery of DNA synthesis following cycloheximide removal without a concomitant recovery of etoposide cytotoxicity (compare Figs. 1 and 4) and the relatively limited effect of Ara-C (data not shown) or

aphidicolin which also blocks cell cycle traverse. Undoubtedly, both of these observations are subject to other interpretations, and the mechanism for the effect of cycloheximide remains to be determined conclusively. It is clear, however, that protein synthesis inhibition can directly or indirectly dissociate DNA cleavable complex formation from cytotoxicity, and this in and of itself should provide an interesting tool for elucidating the mechanism of cell death by this important class of agents.

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